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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 **:** C12N 15/13, C12P 21/08 C12N 5/20, A61K 39/395, 48/00 C12N 5/08, A61K 35/14 G01N 33/577, 33/68	A1	**(11) International Publication Number:** **WO 94/09137** **(43) International Publication Date:** 28 April 1994 (28.04.94)

(21) International Application Number: PCT/US93/09620

(22) International Filing Date: 8 October 1993 (08.10.93)

(30) Priority data:
07/961,602 15 October 1992 (15.10.92) US

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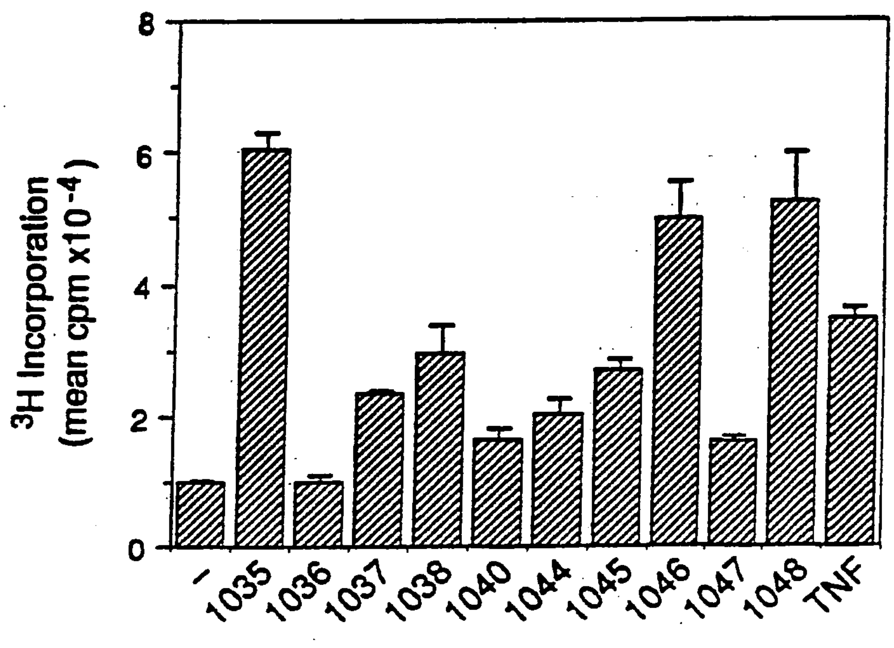
(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ANTIBODIES AGAINST TYPE 2 TUMOR NECROSIS FACTOR RECEPTOR



(57) Abstract

The present invention relates to antibodies against type 2 tumor necrosis factor receptor (TN-R2). More particularly, the invention concerns monoclonal antibodies against human TN-R2 which mimic the T-cell proliferation stimulating activity of TN. The use of such antibodies for the stimulation of human T-cell proliferation is encompassed by the present invention.

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ANTIBODIES AGAINST TYPE 2 TUMOR NECROSIS FACTOR RECEPTOR

Field of the Invention

The present invention relates to antibodies against type 2 tumor necrosis factor receptor (TNF-R2). More particularly, the invention concerns monoclonal antibodies against human TNF-R2 which mimic the T-cell proliferation stimulating activity of TNF. The use of such antibodies for the stimulation of human T-cell proliferation is encompassed by the present invention.

Background of the Invention

Tumor necrosis factor (TNF, also referred to as TNF-α) is a potent cytokine produced mainly by activated macrophages and a few other cell types. The large number of biological effects elicited by TNF include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammatory, immunoregulatory, proliferative, and antiviral responses [reviewed in Goeddel, D.V. *et al.*, Cold Spring Harbor Symposia on Quantitative Biology 51, 597-609 (1986); Beutler, B. and Cerami, A., Ann. Rev. Biochem. 57, 505-518 (1988); Old, L.J., Sci. Am. 258(5), 59-75 (1988); Fiers, W. FEBS Lett. 285(2), 199-212 (1991)]. The literature has reported that TNF and other cytokines such as IL-1 may protect against the deleterious effects of ionizing radiation produced during a course of radiotherapy, such as denaturation of enzymes, lipid peroxidation, and DNA damage (Neta *et al.*, J. Immunol. 136(7): 2483, (1987); Neta *et al.*, Lymphokine Res. 5 *et al.*, supra; Neta *et al.*, Fed. Proc. 46: 1200 (abstract), (1987); Urbaschek *et al.*, Lymphokine Res. 6: 179 (1987); U.S. Patent No. 4,861,587; Neta *et al.*, J. Immunol. 140: 108 (1988). A related molecule, lymphotoxin (LT, also referred to as TNF-β), that is produced by activated lymphocytes shows a similar but not identical spectrum of biological activities as TNF (see, e.g. Goeddel, D.V. *et al.*, supra, and Fiers, W., supra). TNF was described by Pennica *et al.*, Nature 312, 721 (1984); LT was described by Gray *et al.*, Nature 312, 724 (1984).

The first step in the induction of the various cellular responses mediated by TNF or LT is their binding to specific cell surface receptors. Two distinct TNF receptors of approximately 55-kDa (TNF-R1) and 75-kDa (TNF-R2) have been identified [Hohman, H.P. *et al.*, J. Biol. Chem. 264, 14927-14934 (1989); Brockhaus, M. *et al.*, Proc. Natl. Acad. Sci. USA 87, 3127-3131 (1990)], and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized [Loetscher, H. *et al.*, Cell 61, 351 (1990); Schall, T.J. *et al.*, Cell 61, 361 (1990) (11-15); Smith, C.A. *et al.*, Science 248, 1019 (1990); Lewis, M. *et al.*, Proc. Natl. Acad. Sci. USA 88, 2830-2834 (1991); Goodwin, R.G. *et al.*, Mol. Cell. Biol. 11, 3020-3026 (1991)]. Both TNF-Rs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally also as soluble TNF-binding proteins [Nophar,

Y. *et al.*, EMBO J. 9, 3269 (1990); and Kohno, T. *et al.*, Proc. Natl. Acad. Sci. U.S.A. 87, 8331 (1990)]]. The amino acid sequence of human TNF-R1 and the underlying nucleotide sequence are disclosed in EP 417,563 (published 20 March 1991), whereas EP 418,014 (published 20 March 1991) discloses the amino acid and nucleotide sequences of human TNF-R2.

Although not yet systematically investigated, the majority of cell types and tissues appear to express both TNF receptors. In attempting to understand the individual roles of the two TNF receptors, several groups have generated poly- and monoclonal antibodies (mAbs) that are specific for either TNF-R1 or TNF-R2.

It has been observed that both polyclonal and monoclonal antibodies directed against TNF-R1 can act as specific agonists for this receptor and elicit several TNF activities such as cytotoxicity, fibroblast proliferation, resistance to chlamidiae, and synthesis of prostaglandin E_2 [Engelman, H. *et al.*, J. Biol. Chem. 265, 14497-14504 (1990); Espevik, T. *et al.*, J. Exp. Med. 171, 415-426 (1990); Shalaby, M.R. *et al.*, J. Exp. Med. 172, 1517-1520 (1990)].

Polyclonal antibodies to both murine TNF-R1 and TNF-R2 have been developed, and each shown to behave as specific receptor agonists and induce a subset of murine TNF activities. While the murine TNF-R1 was shown to be responsible for signaling cytotoxicity and the induction of several genes, the murine TNF-R2 was shown to be capable of signaling proliferation of primary thymocytes and a cytotoxic T cell line [Tartaglia, L.A. *et al.*, Proc. Natl. Acad. Sci. USA 88, 9292-9296 (1991)]. Monoclonal antibodies against human TNF-R1 that block the binding of TNF to TNF-R1 and antagonize several of the TNF effects have also been described [Espevik, T. *et al.*, Supra; Shalabi, M.R. *et al.*, Supra; Naume, B. *et al.*, J. Immunol. 146, 3035-3048 (1991)].

In addition, several reports described monoclonal antibodies directed against TNF-R2 that can partially antagonize the same TNF responses (such as cytotoxicity and activation of NF-κB) that are induced by TNF-R1 agonists [Shalaby, M.R. *et al.*, Supra; Naume, B. *et al.*, Supra; and Hohman, H.P. *et al.*, J. Biol. Chem. 265, 22409-22417 (1990)].

The foregoing reports suggested that both TNF receptors are active in signal transduction and that there is redundancy in the function of the two receptors. An opposing view was taken by Thoma, B. *et al.*, J. Exp. Med. 172, 1019-1023 (1990) who demonstrated that antagonist antibodies to TNF-R1 could completely block several TNF activities even in cells expressing both receptor types. This led them to propose that TNF-R1 is the biologically relevant TNF receptor, and binding of TNF to TNF-R2 is not sufficient to initiate TNF responses.

The individual roles of the two TNF receptors, and particularly those of TNF-R2, in cell signaling are far from entirely understood. Although the reported effects of the anti-TNF-R2 antagonist antibodies suggest a direct role for TNF-R2 in signal transduction, these effects were quite small and occurred at only low TNF concentrations. These data, therefore, appear

to be more consistent with a model in which TNF-R2 is participating as a minor accessory component to TNF-R1 in the signaling of responses like cytotoxicity and activation of NF-kB [Tartaglia, L.A. and Goeddel, D.V., Imm. Today 13, 151-153 (1992)].

It is an object of the present invention to identify biological activities specifically mediated by human TNF-R2.

It is a further object of the present invention to provide agonist monoclonal antibodies to human TNF-R2 showing immunoregulatory activity.

In is a still further object to provide agonist monoclonal antibodies to human TNF-R2 capable of the stimulation of human T cell proliferation.

It is another object to provide a method for the treatment of diseases or physiological conditions where the enhancement of T cell proliferation is desirable.

It is yet another object to provide bispecific anti-TNFR2 antibodies.

It is still another object to provide a method for detecting human type 2 TNF receptors in a source suspected of containing them.

These and further objects will be apparent for those skilled in the art.

Summary of the Invention

The present invention is based on the experimental finding that poly- and monoclonal antibodies directed against human TNF-R2 strongly stimulate human thymocyte proliferation, whereas antibodies to human TNF-R1 have no such effect. These results indicate that although the two human TNF receptors are both active in signal transduction, they are able to mediate distinct cellular responses. Specifically, TNF induces human T cell proliferation exclusively via TNF-R2 and antibodies against human TNF-R2 can act as receptor agonists. This finding is unexpected, since thymocyte proliferation is the first TNF response that can be initiated by agonist antibodies to human TNF-R2 and not to human TNF-R1. Furthermore, the ability of monoclonal antibodies against TNF-R2 to act directly as agonists is surprising, since the large majority of monoclonal antibodies to TNF-R1 require either cross-linking with a second antibody or a combination of at least two monoclonal antibodies for agonist activity.

In one aspect, the present invention concerns a monoclonal antibody composition which reacts physiologically with a TNF-R2 and exhibits immune regulatory activity. The receptor is preferably human TNF-R2, and the monoclonal antibody composition is preferably capable of stimulating T-cell proliferation, and most preferably is capable of binding to substantially the same human TNF-R2 epitope as that recognized by an antibody selected from the group consisting of monoclonal antibodies 1035, 1036, 1037, 1038, 1039, 1040, 1043, 1044, 1045, 1046, 1047, and 1048.

In another aspect, the invention concerns an isolated nucleic acid encoding a monoclonal antibody as hereinbefore defined.

In a further aspect, the invention concerns a hybridoma cell line which produces a monoclonal antibody as hereinabove defined.

In a still further aspect, the invention concerns a pharmaceutical composition comprising a monoclonal antibody as hereinabove defined in an amount effective in stimulating T-cell proliferation.

In yet another aspect, the invention concerns a bispecific antibody specific for a human TNF-R2 and for a T-cell surface protein. The T-cell surface protein preferably is a CD antigen, more preferably CD1, CD2, CD3, CD4, CD5, CD6, CD8, CD11a, CD11b, CD11c, or CD18; VLA-4 or LFA-1. In another preferred embodiment, the T-cell surface protein is an interleukin-2 receptor (IL-2R), when specificity for IL-2R may, for example, be provided by an IL-2 amino acid sequence. In a further preferred group of bispecific antibodies, the T-cell surface protein is a ganglioside, such as G_{D3}, and the ganglioside-specificity is from an antibody to the ganglioside. The bispecific antibodies herein may comprise an Fc domain having an immunoglobulin effector function, which may be from an IgA, IgD, IgE, IgG or IgM immunoglobulin.

In a different aspect, the invention concerns a method for stimulating human T-cell proliferation comprising administering to a human patient is need of such treatment a physiologically effective amount of a monoclonal antibody which reacts immunologically with a human TNF-R2. If desired, the anti-TNF-R2 monoclonal antibody is coadministered with a further T-cell mitogen, such as IL-2. The physiological conditions to be treated include, but are not limited to, T-cell mediated autoimmune disorders, immunodeficiencies, HIV, graft-versus-host disease, and potential of allograft rejection. In a particular embodiment, the administration may be by gene transfer.

In a further aspect, the invention concerns a method for stimulating human T-cell proliferation comprising growing a human T-cell culture in the presence of an effective amount of a monoclonal antibody which reacts immunologically with a human TNF-R2.

In a still further aspect, the invention concerns a method for detecting a human TNF-R2 by contacting a source suspected of containing such receptor with a detectably labeled monoclonal antibody which reacts immunologically with a native human TNF-R2, and determining whether the antibody binds to the source.

Brief Description of the Drawings

Figure 1. Proliferative response of human thymocytes to (A) TNF and (B) rabbit polyclonal antibodies directed against the human TNF receptors. (■), anti-TNF-R1; (•), anti-TNF-R2; (□), prebleed TNF-R1; (o), prebleed TNF-R2. Thymocytes were cultured for 60 h with 2 μg/ml Con A and the indicated concentrations of TNF or anti-TNF-R antibody. 3H incorporation was determined as described in the Example. The amount of 3H incorporation

in thymocytes treated with Con A alone is indicated by a dashed line. No enhancement of proliferation was observed in the absence of Con A.

Figure 2. Proliferative response of human thymocytes to TNF or LT. (o), TNF; (•), LT. Thymocytes were cultured for 60 h with 2 μg/ml Con A and the indicated concentrations of TNF or LT. 3H incorporation was determined as described in Materials and Methods. The amount of 3H incorporation in thymocytes treated with Con A alone is indicated by a dashed line.

Figure 3. Proliferative response of human thymocytes to (A) TNF (B) polyclonal antibodies directed against the two human TNF receptors: (•), TNF-R1 polyclonal anti-sera; (o) TNF-R2 polyclonal antisera or (C) monoclonal antibodies directed against human TNF-R2: (o), anti-TNF-R2 Mab #1035; (■), #1036; (□), #1037; (▵), #1038; (▾), #1040; (■), #1044; (▴), #1045; (□), #1046; (▿), #1047; (•), #1048; (+), anti-mouse IgG. Thymocytes were cultured for 60 h with 2 μg/ml ConA and the indicated concentrations of TNF or anti-TNF-R antibodies. 3H incorporation was determined as described in Materials and Methods. The amount of 3H incorporation in thymocytes treated with Con A alone is indicated by dashed lines.

Figure 4. Proliferative response of human thymocytes to TNF and mAbs directed against human TNF-R2. Thymocytes were cultured for 60 h with 2 μg/ml ConA and 10 μg/ml anti-TNF-R2 Mabs or control mouse IgG (-). The maximal response observed with a titration of TNF in a parallel assay is shown for comparison. 3H incorporation was determined as described in the Example. Results are the mean of triplicate determinations (+/- SD).

Figure 5. Proliferative response of human PBMC to rabbit polyclonal antibodies directed against the human TNF receptors. (o), anti-TNF-R1; (•), anti-TNF-R2; (□), prebleed TNF-R1; (■), prebleed TNF-R2. PBMC were cultured for 5 days with PHA-P and the indicated concentrations of anti-TNF-R antibody. 3H incorporation was determined as described in Materials and Methods. The amount of 3H incorporation in PBMC treated with PHA-P alone is indicated by a dashed line. No enhancement of proliferation was observed in the absence of PHA-P.

Detailed Description of the Invention

A. Definitions

The terms "type 2 TNF receptor" and "TNF-R2" are used interchangeably, and refer to a family of polypeptide molecules that comprise any naturally occurring (native) type 2 TNF receptor from any animal species, and amino acid sequence and glycosylation variants of such receptors, provided that the DNA sequences encoding such variants are capable of hybridizing, under stringent conditions, to a DNA sequence encoding a native type 2 TNF receptor, and that they retain the ability to bind TNF. The amino acid sequence variants preferably share at least about 65% sequence homology, more preferably at least about 75%

sequence homology, even more preferably at least about 85% sequence homology, most preferably at least about 90% sequence homology with any domain, and preferably with the ligand binding domain(s), of the native full-length TNF-R2 amino acid sequence from the same (human or non-human) animal species.

The terms "human type 2 TNF receptor" and "human TNF-R2", which are used interchangeably, refer to a family of polypeptide molecules that comprise the full-length, native human TNF-R2 having the amino acid sequence disclosed in EP 418,014 (published 20 March 1991), and its amino acid sequence variants, provided that the DNAs encoding such variants are capable of hybridizing under stringent conditions with the DNA encoding the native amino acid sequence, and that they retain the ability to bind TNF. This definition specifically encompasses soluble forms of native full-length human TNF-R2, from natural sources, synthetically produced in vitro or obtained by genetic manipulation including methods of recombinant DNA technology. The amino acid sequence variants preferably share at least about 65% sequence homology, more preferably at least about 75% sequence homology, even more preferably at least about 85% sequence homology, most preferably at least about 90% sequence homology with any domain, and preferably with the ligand binding domain(s), of the native full-length human TNF-R2 amino acid sequence. The definition specifically covers variously glycosylated and unglycosylated forms of native full-length human TNF-R2 and of its amino acid sequence variants.

The "stringent conditions" are overnight incubation at 42 °C in a solution comprising: 20% formamide, 5xSSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA.

The term "native human type 2 TNF receptor" or native human TNF-R2" is used to refer to the mature full-length native human TNF-R2 as disclosed in EP 418,014 (published 20 March 1991), and any naturally occurring fragment or derivative thereof provided that it retains the ability to bind TNF.

The terms "amino acid" and "amino acids" refer to all naturally occurring L-α-amino acids. The amino acids are identified by either the single-letter or three-letter designations:

Asp	D	aspartic acid	Ile	I	isoleucine
Thr	T	threonine	Leu	L	leucine
Ser	S	serine	Tyr	Y	tyrosine
Glu	E	glutamic acid	Phe	F	phenylalanine
Pro	P	proline	His	H	histidine
Gly	G	glycine	Lys	K	lysine
Ala	A	alanine	Arg	R	arginine
Cys	C	cysteine	Trp	W	tryptophan

Val	V	valine	Gln	Q	glutamine
Met	M	methionine	Asn	N	asparagine

These amino acids may be classified according to the chemical composition and properties of their side chains. They are broadly classified into two groups, charged and uncharged. Each of these groups is divided into subgroups to classify the amino acids more accurately:

I. Charged Amino Acids

Acidic Residues: aspartic acid, glutamic acid

Basic Residues: lysine, arginine, histidine

II. Uncharged Amino Acids

Hydrophilic Residues: serine, threonine, asparagine, glutamine

Aliphatic Residues: glycine, alanine, valine, leucine, isoleucine

Non-polar Residues: cysteine, methionine, proline

Aromatic Residues: phenylalanine, tyrosine, tryptophan

The term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a native amino acid sequence.

"Homology" is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of their native counterparts after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

The term "glycosylation variant" is used to refer to a glycoprotein having a glycosylation profile different from that of a native counterpart. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate

moiety to the side-chain of an asparagine residue. The tripeptide sequences, asparagine-X-serine and asparagine-X-threonine, wherein X is any amino acid except proline, are recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be involved in O-linked glycosylation. Any difference in the location and/or nature of the carbohydrate moieties present in a TNF-R2 protein as compared to its native counterpart is within the scope herein.

Antibodies (Abs) and immunoglobulins (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one and (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains [Clothia *et al.*, J. Mol. Biol. 186, 651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA 82, 4592-4596 (1985)].

The variability is not evenly distributed through the variable regions of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable regions. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies [see Kabat, E.A. *et al.*, Sequences of Proteins of Immunological Interest National Institute of Health, Bethesda, MD (1987)]. The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

Papain digestion of antibodies produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (C_H1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other, chemical couplings of antibody fragments are also known.

The light chains of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant region of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3, and IgG-4. The heavy chain constant regions that correspond to the different classes of immunoglobulins are called α, delta, epsilon, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "antibody" is used herein in the broadest sense and specifically covers single monoclonal antibodies, immunoglobulin chains or fragments thereof, which react immunologically with a type 2 TNF receptor (TNF-R2) and exhibit immune regulatory, preferably T-cell proliferation stimulating activity, as well as anti-TNF-R2 antibody compositions with polyepitopic specificity, which have such properties.

The term "monoclonal antibody" as used herein refers to an antibody (as hereinabove defined) obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly

specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins.

"Humanized" forms of non-human (e.g. murine) antibodies are immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance.

The monoclonal antibodies herein include hybrid (chimeric) and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-TNF-R2 antibody with a constant domain (e.g. "humanized" antibodies), only one of which is directed against TNF-R2, or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv), so long as they exhibit immune regulatory, and preferably T-cell proliferation stimulating, activity. [See, e.g. Cabilly, et al., U.S. Pat. No. 4,816,567; Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New York, 1987).]

For "chimeric" and "humanized" antibodies see, for example, U.S. Patent No. 4,816,567; WO 91/09968; EP 452,508; and WO 91/16927).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method.

The antibodies herein may be of any immunoglobulin class or isotype, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA, IgE, IgD or IgM. If an immunoglobulin effector function is desirable, the antibodies herein typically retain at least functionally active hinge, CH2 and CH3 domains of the constant region of the heavy chain of an immunoglobulin.

The phrases "agonist antibody" or "agonist anti-TNF-R2 antibody" as used herein refer to antibodies to the type 2 TNF receptor that are able to mimic the immune regulatory activity, and specifically the T-cell proliferation stimulating activity of TNF.

As used herein the phrase "bispecific antibody" designates antibodies (as hereinabove defined) having at least two binding specificities, one of which is an antigen binding site of an anti-TNF-R2 antibody. Bispecific antibodies can generally be assembled as hetero-multimers, and particularly as hetero-dimers, -trimers or -tetramers, essentially as disclosed in WO 89/02922 (published 6 April 1989), in EP 314,317 (published 3 May 1989), and in U.S. Patent No. 5,116,964 issued 2 May 1992.

T-cells, also referred to as T-lymphocytes, are specialized white blood cells that carry out specific cellular immune responses by interacting with antigens through membrane-anchored T-cell receptors. T cells arise from stem cells located in the bone marrow (the primary lymphoid organ), and mature in the thymus. T- cells at this stage of development are referred to as "thymocytes" as opposed to mature T-cells, located in the secondary lymphoid organs, including the peripheral lymph nodes, the tonsils, the spleen, and Peyer's patches. T-cells can be grouped in three broad classes which differ greatly in their responses to antigen. Two types of T-cells regulate the immune response while the third type of T-cells carries out an effector function. Helper T-cells (T_H cells) generally increase the response to antigen by secreting soluble regulators, cytokines, which act on other T-cells and on the other arms of the immune system. For example, helper cells provide growth factors necessary for B-cell immunoglobulin production in response to most antigens. Suppressor T-cells (T_S cells) inhibit the immune response, whereas cytotoxic T-cell (also referred to as cytotoxic T lymphocytes or CTLs) kill cells which carry foreign antigen on their surface. These cells do not normally regulate the response of other leukocytes to foreign invasion but they are important for the elimination of virus infected cells from the body. The term "T-cell" as used throughout the specification refers to all types and subpopulations of T-cells, such as helper T-cells (T_H cells), cytotoxic T lymphocytes (CTLs), suppressor T-cells (T_S cells), tumor infiltrating lymphocytes (TILs), from any source, in any stage of development (specifically including thymocytes and mature T-cells).

The phrase "immune regulatory activity" is used herein to refer to any biological activity affecting the differentiation, division, proliferation, or activation of T-cells. It will be understood that this (T-cell associated) immune regulatory activity may indirectly affect B-cell associated immune responses. For example, B-cell differentiation, division, and antibody secretion may be promoted by means of T_H cells, whereas B-cell proliferation and terminal differentiation may be suppressed by means of T_S cells.

The CD T-cell surface glycoproteins are members of the immunoglobulin gene superfamily which are expressed on the surface of various populations of T lymphocytes. They include CD1, CD2, CD3, CD4, CD5, CD6, CD8, CD11a, CD11b, CD11c, and CD18.

The CD antigens are known in the art, and have been identified to have various functions in cell-cell interactions and various T-cell functions. For example, CD2 is an approximately 55 kD glycoprotein found on the surface of thymocytes and mature T-cells [Springer *et al.*, Ann. Rev. Immunol. 5, 223-252 (1987)]. CD2 binds a widely distributed cell surface protein, LFA-3. The two proteins form a cell-cell adhesion system which can cause thymocytes to adhere to thymic epithelium, and which aids in the adhesion of mature T-cells to antigen presenting on target cells, and has also been identified as playing a direct role in T-cell function. CD4 and CD8 (also known as T4/Leu3 and T8/Leu2) are nonpolymorphic members of the immunoglobulin gene superfamily which are expressed on the surface of functionally distinct populations of T lymphocytes. These molecules have been identified as essential for effective cell-cell interactions, resulting either in target cell lysis or in activation of T-lymphocytes [see Littman, Ann. Rev. Immunol. 5, 561-584 (1987)]. CD4 was shown to be the receptor for the human immunodeficiency virus, HIV [reviewed in Capon and Ward, Annu. Rev. Immunol. 9, 649-678 (1991)]. As the hallmark of HIV infection is the specific destruction of the $CD4^+$ T cells, and the progression of infected individuals to AIDS closely parallels their decline in $CD4^+$ T-cell number [Lane, H. and Fauci, A., Annu. Rev. Immunol. 3, 477-500 (1985)], it was proposed that the interaction of the HIV-1 envelope glycoprotein, gp120 with the CD4 receptor, either by direct HIV infection of $CD4^+$ cells or otherwise, underlies the killing of $CD4^+$ cells.

The term "CD", with or without the appropriate number (e.g. CD1, CD2, CD3, CD4, etc.) is used to designate a native-sequence CD molecule, or any fragment or derivative thereof, whether isolated from natural source, chemically synthesized or produced by methods of recombinant DNA technology, provided that it retains the biological activity in kind of the corresponding native CD protein.

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological property, as screened for in the originally transformed cell, are included.

"Transformation" means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integration.

"Transfection" refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed.

The terms "transformed host cell" and "transformed" refer to the introduction of DNA into a cell. The cell is termed a "host cell", and it may be a prokaryotic or a eukaryotic cell. Typical prokaryotic host cells include various strains of E. coli. Typical eukaryotic host cells are mammalian, such as Chinese hamster ovary cells or human embryonic kidney 293 cells. The introduced DNA is usually in the form of a vector containing an inserted piece of DNA.

The introduced DNA sequence may be from the same species as the host cell or a different species from the host cell, or it may be a hybrid DNA sequence, containing some foreign and some homologous DNA.

The terms "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods [such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as those described in EP 266,032, published 4 May 1988, or via deoxynucleoside H-phosphanate intermediates as described by Froehler *et al.*, Nucl. Acids Res. 14, 5399 (1986)]. They are then purified on polyacrylamide gels.

B. Methods of Producing and Characterizing Monoclonal Antibodies

The following is a brief discussion of certain commonly used techniques that can be used for making the antibodies of the present invention. Further details of these and similar techniques are found in general textbooks, such as, for example, Cabilly, *et al.*, U. S. Patent No. 4,816,567; Mage & Lamoyi, supra; Sambrook *et al.*, Molecular Cloning: A laboratory Manual (Second Edition, Cold Spring Harbor Laboratory Press, New York, 1989; and Current Protocols in Molecular Biology, Ausubel *et al.* eds., Green Publishing Associates and Wiley-Interscience, 1991.

The anti-TNF-R2 antibodies of the present invention may be produced by any method known in the art. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods [Cabilly, et al., supra].

In the hybridoma method, a mouse or other appropriate host animal, such as hamster is immunized with a human TNF-R2 protein by subcutaneous, intraperitoneal, or intramuscular routes to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, J. Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)].

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies. Kozbor, J. Immunol. 133:3001 (1984). Brodeur, et al., Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against TNF-R2. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The monoclonal antibodies for use in the method and compositions of the invention are those that preferentially immunoprecipitate TNF-R2 that is present in a test sample, or that preferentially bind to TNF-R2 in a binding assay, and are capable of stimulating T-cell growth in an experimental model of T-cell proliferation, such as the thymocyte proliferation assay or the peripheral T-cell proliferation assay disclosed in the Example.

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, J., Supra). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Methods for purification of monoclonal antibodies are well known in the art, and are, for example disclosed in Unit 11.11 of "Current Protocols in Molecular Biology", supra, and in the references cited therein.

The amount of a specific antibody present in a hybridoma supernatant can be quantitated by either solid-phase radioimmunoassay (RIA) or by direct enzyme-linked immunoabsorbent assay (ELISA). In the solid-phase radioimmunoassay, serially diluted antiserum is incubated in microtiter wells previously coated with TNF-R2. Bound antibody is detected by employing ^{125}I-labeled anti-immunoglobulin antibodies. The amount of the specific antibody in the antiserum is then determined from a standard curve generated with a specific antibody of known concentration. The unknown antiserum and the standard antibody are assayed in parallel. Protocols for the RIA procedure as used for isotype determination, and the ELISA procedure are, for example, available from Section V of "Current Protocols in Molecular Biology", supra, and from the references cited therein.

The monoclonal antibodies herein are preferably against epitopes within the extracellular domain of human TNF-R2, and preferably bind to the same epitope as any of the antibodies specifically disclosed herein. To determine whether a monoclonal antibody has the same specificity as any of the anti-TNF-R2 antibodies specifically disclosed (e.g. antibody # 1035 having the ATCC deposit number HB 11150), one can, for example, use the competitive ELISA binding assay disclosed in the Example.

DNA encoding the monoclonal antibodies useful in the method of the invention is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese Hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The immunoglobulin polypeptides of this invention are made in conventional fashion, i.e., modifications of amino acid sequence are accomplished by commonly available DNA mutagenesis methods such as PCR amplification using primers bearing the mutants, or by M13 mutagenesis, followed by expression of the mutated DNA in recombinant host cells. The polypeptides also can be made by Merrifield or other *in vitro* methods of synthesis if they are sufficiently small (generally, under about 100 residues). However, the polypeptides preferably are made by recombinant methods. Section of recombinant host cells, vectors, culture conditions and other parameters are not believed to be critical. In general, hosts, vectors and methods heretofore used in the recombinant expression of immunoglobulins (generally, IgGs) are also useful for the preparation of the polypeptide sequences of this invention. Preferably, mammalian cells such as myelomas, CHO, COS, 293s and the like are employed as hosts, and the vectors are constructed for secretory expression of the polypeptide. Recombinant expression systems facilitate the preparation of functional immunoglobulin variants containing TNF-R2-specific sequences since the host cells can be transformed with DNA encoding one

heavy chain containing the TNF-R2-specific sequences and one light chain, each of which contains a variable domain for binding a first antigen, and an immunoglobulin that binds antigen and TNF-R2 recovered. Similarly, the same process is used with DNA encoding in addition another heavy chain containing the TNF-R2-specific domain and another light chain, each of which contain a variable domain for binding a second antigen, and a bivalent immunoglobulin recovered. Properly assembled immunoglobulin analogues are recovered by affinity chromatography on a matrix containing the two antigen(s).

The polypeptides of this invention are recovered from lysed recombinant cell culture or (when secreted) the culture supernatant. Substantial purification is achieved by passing cell free extracts which contain the polypeptides over an immobilized TNF-R2 affinity matrix. Other methods heretofore used to purify other appropriate immunoglobulins are equally acceptable here, including immunoaffinity and (when appropriate) absorption on immobilized antigen.

Polypeptides of this invention which contain short sequences preferably are prepared using solid-phase synthesis, e.g. the method of Merrifield, J. Am. Chem. Soc., 85: 2149 (1963). However, other equivalent chemical syntheses known in the art are acceptable. The recombinant or *in vitro* synthesized polypeptides then are cross-linked to matrices (for use in diagnostic or preparatory procedures) or are placed into conformationally restrained structures. Known cyclizing procedures such as those described in PCT 90/01331 or Lys/Asp cyclization using Nα-Boc-amino acids on solid-phase support with Fmoc/9-fluorenylmethyl (Ofm) side-chain protection for Lys/Asp, followed by piperidine treatment and cyclization, are useful. Methods which depend upon cross-linking or cyclization through residue side chains may require that an extraneous residue be inserted at the C and/or N terminus of the AB-B or beta stand D domains, as the case may be, to provide a suitable cyclizing or cross-linking site.

Glu and Lys side chains also have been crosslinked in preparing cyclic or bicyclic peptides: the peptide is synthesized by solid phase chemistry on a p-methylbenzhydrylamine resin, the peptide is cleaved from the resin and deprotected. The cyclic peptide is formed using diphenylyphosphorylazide in diluted methylformamide. For an alternative procedure, see Schiller *et al.*, Peptide Protein Res. 25: 171-177 (1985). See also U.S. Patent 4,547,489.

Disulfide crosslinked or cyclized peptides are generated by conventional methods. The method of Pelton *et al.*, J. Med. Chem. 29: 2370-2375 (1986) is suitable. Also useful are thiomethylene bridges (Tetrahedron Letters 25: 2067-2068 (1986). See also Cody *et al.*, J. Med. Chem. 28: 583 (1985). The C390 residue found in the C-terminal sequence of the AB-B domain is useful in dross-linking or cyclizing this domain.

Typically, extraneous residues which are to participate in cyclization or cross-linking are inserted at the N- and C-termini of the chosen AB-B or beta strand D sequence as part of the synthesis of the polypeptide precursor to be employed in the procedure. The desired cyclic or cross-linked peptides are purified by gel filtration followed by reversed-phase high pressure

liquid chromatography or other conventional procedures. The peptides are sterilized by 0.2 μm filtration and formulated into conventional pharmacologically acceptable vehicles.

The compounds described in this invention may be the free acid or base or converted to salts of various inorganic and organic acids and bases. Such salts are within the scope of this invention. Examples of such salts include ammonium, metal salts like sodium, potassium, calcium and magnesium; salts with organic bases like dicyclohexylamineN-methyl-D-glucamine and the like; and salts with amino acids such as arginine or lysine. Salts with inorganic and organic acids may be like prepared, for example, using hydrochloric, hydrobromic, sulfuric, phosphoric, trifluoroacetic, methanesulfonic, maleic, fumaric and the like. Non-toxic and physiologically compatible salts are particularly useful although other less desirable salts may have used in the processes of isolation and purification.

A number of methods are useful for the preparation of the salts described above and are known to those skilled in the art. For example, reaction of the free acid or free base form of a compound of Formula I with one or more molar equivalents of the desired acid or base in a solvent or solvent mixture in which the salt is insoluble; or in a solvent like water after which the solvent is removed by evaporation, distillation or freeze drying. Alternatively, the free acid or base form of the product may be passed over an ion exchange resin to form the desired salt, or one salt form of the product may be converted to another using the same general process.

Additional pharmaceutical methods may be employed to control the duration of action of the polypeptides of this invention. Controlled release preparations are achieved through the use of polymers which complex with or absorb the subject polypeptides. Controlled delivery is achieved by formulating the polypeptides into appropriate macromolecular articles (for example, those prepared from polyesters, polyamino acids, polyvinyl, polypyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or polyamine sulfate).

Alternatively, instead of entrapping the polypeptides in polymeric matrices, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization. Hydroxymethylcellulose or gelatin microcapsules and poly-(methylmethacrylate) microcapsules, respectively, are useful, as are in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules). See Remington's Pharmaceutical Sciences (1980).

The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [Morrison, et al., Proc. Nat. Acad. Sci. 81:6851 (1984)], or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-TNF-R2 monoclonal antibody herein.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent (bispecific) antibody comprising one antigen-combining site having specificity for TNF-R2, preferably human TNF-R2, and another antigen-combining site having specificity for a different antigen.

The second specificity is preferably provided by an amino acid sequence specific for a T-cell surface protein such as VLA-4, LFA-1 or a CD antigen. Such amino acid sequence may, for example, include the antibody-antigen combining site of an antibody to a CD antigen (a T-cell marker), or an amino acid sequence specifically binding to an interleukin receptor, e.g. interleukin-2 receptor (IL-2R), or interleukin-4 receptor (IL-4R), such as an anti-IL-2R or anti-IL-4 antibody sequence or an IL-2 or IL-4 amino acid sequence, or an amino acid sequence specifically binding to a ganglioside, such as the antibody-antigen combining site of an anti-ganglioside antibody.

If the second specificity is provided by an antibody to a CD antigen, such antibody may, for example, be an anti-CD1, anti-CD2, anti-CD3, anti-CD4, anti-CD5, anti-CD6, anti-CD8, anti-CD11a, anti-CD11b, anti-CD11c, or anti-CD18 monoclonal antibody, and preferably an anti-CD2, anti-CD4, anti-CD8, anti-CD18, anti-CD11a, anti-CD11b, or anti-CD11c monoclonal antibody. Such monoclonal antibodies are known in the art, and are commercially available (e.g. OKT-2, OKT-3, OKT4-, OKT-6, OKT-8, OKT-11 can be purchased from Ortho Diagnostic Systems, Raritan, N.J.), or can be easily produced by techniques known in the art.

The disialoganglioside G_{D3} has been shown to be expressed on cell types derived from the neuroectoderm, including melanocytes, adrenal medullary cells, glia, neurons, islet cells of the pancreas, human thymocytes and peripheral blood lymphocytes. Monoclonal antibodies against this ganglioside have been demonstrated to stimulate T-cell proliferation in human thymocytes and peripheral blood lymphocytes (see United States Patent No. 5,104,652 issued 14 April 1992). A hybridoma cell line (M-18) producing one of these antibodies (R24) has been deposited and is available from the American Type Culture Collection (ATCC) under accession number HB 8445.

Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents or adventitious oxidants such as dissolved oxygen. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

The monoclonal antibodies included within the scope of the invention belong to a subclass or isotype that, upon complexing with a TNF-R2 receptor, induce a proliferative response of human thymocytes. The human monoclonal antibodies used in the experiments disclosed herein, are of the IgG class. Under certain circumstances, monoclonal antibodies

of one class or isotype might be more preferable than those of another in terms of their diagnostic, analytical or therapeutic efficacy. It is known that specific receptors on placental cells recognize the constant region of IgG (and only IgG) molecules, causing them to be ingested by endocytosis, shuttled across the cell, and released into the fetal blood on the other side of the cell. As IgG is the only immunoglobulin that can cross the placenta to travel from mother to fetus during pregnancy, IgG constant domains must be used in antibodies designed for the perinatal treatment of fetus. If a longer plasma half-life of the antibody is desirable, immunoglobulins of IgG-1, IgG-2 and IgG-4 isotypes are good candidates, as they all have *in vivo* half-lives of 21 days, as opposed to IgG-3 which has an *in vivo* half-life of 7 days. Further differences that might be advantages or detriments in certain situations are, for example, in complement activation. IgG-1, IgG-2 and IgG-3 all activate complement, however, IgG-2 is significantly weaker at complement activation than IgG-1 and does not bind to Fc receptors on mononuclear cells or neutrophils, while IgG-3 shows better complement activation than IgG-1. IgG-1 has only four serologically-defined allotypic sites, two of which (G1m1 and 2) are in the Fc portion; for two of these sites (G1m1 and 17) one allotype is non-immunogenic. In contrast, there are 12 serologically defined allotypes in IgG-3, all of which are in the Fc portion, only three of which (G3m5, 11 and 21) have an allotype which is non-immunogenic. Thus, for repeated and long-term therapeutic applications, antibodies with IgG-1 derived constant domain sequences are preferred. It is possible to use immunoglobulins from different classes or isotypes in the two arms of a (bispecific) antibody molecule. It is further possible to combine sequences from various immunoglobulin classes or isotypes in the same arm of the antibody molecule. For example, constructs in which the hinge of IgG-1 is replaced with that of IgG-3 are fully functional. In general, particular isotypes of a monoclonal antibody can be prepared directly, by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection technique to isolate class-switch variants [Steplewski *et al.*, Proc. Natl. Acad. Sci. USA 82, 8653 (1985); Spira *et al.*, J. Immunol. Meth. 74, 307 (1984)]. Alternatively, monoclonal antibodies with any desired isotype can be readily prepared by techniques of recombinant DNA technology.

C. Pharmaceutical Compositions and Administration of anti-TNF-R2 Monoclonal Antibodies

The monoclonal antibodies of the present invention can be advantageously used in the treatment of conditions where the stimulation of T-cell proliferation is beneficial. Such conditions include, for example, the treatment of malignant tumors, T-cell mediated autoimmune diseases, HIV, graft-versus-host disease, and potential of allograft rejection. For example, a certain class of T-cells, called tumor infiltrating lymphocytes (TILs), has been successfully used in the treatment of advanced malignant melanoma in human patients. TILs are isolated directly from the tumor cells, and are then grown to large numbers in tissue

culture in the presence of a T-cell mitogen, such as IL-2. After expansion in culture several thousand times, approximately 2 x 10^{11} TILs are given to the patient intravenously in addition to high doses of IL-2. The monoclonal antibodies of the present invention can be used in this protocol, alone or in combination with other T-cell mitogens, such as IL-2, IL-4, to stimulate TIL growth in cell culture and/or to treat cancer patients. For details of the protocol see Rosenberg, S. *et al.*, N. Eng. J. Med. 319, 1676 (1988).

The antibodies disclosed herein may be administered to a mammal, preferably a human, in a pharmaceutically acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes.

Such dosage forms encompass pharmaceutically acceptable carriers that are inherently nontoxic and nontherapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and polyethylene glycol. Carriers for topical or gel-based forms of antibody include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, polyethylene glycol, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, and sublingual tablets. The antibody will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Pharmaceutical compositions may be prepared and formulated in dosage forms by methods known in the art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 15th Edition 1975.

Depending on the type and severity of the disease, from about 0.001 mg/kg to about 1000 mg/kg, more preferably about 0.01 mg to 100 mg/kg, more preferably about 0.010 to 20 mg/kg of antibody might be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is repeated until a desired suppression of disease symptoms occurs or the desired improvement in the patient's condition is achieved. However, other dosage regimens may also be useful.

D. Gene Marking and Gene Therapy

The anti-TNF-R2 antibodies of the present invention can be advantageously used in various protocols of T-cell associated gene marking and gene therapy.

The first federally approved clinical protocol, initiated in 1989, was for the transfer of certain gene-marked TIL cells into patients with advanced cancer. This protocol is suitable for identifying the subset of TIL effective in killing cancer cells *in vivo*, and to follow the distribution and survival of marked TIL in the patients following administration. According to the NeoR/TIL clinical protocol, TIL are taken from malignant tumors and cultured as hereinabove described. The neomycin resistance gene obtained from E. coli (NeoR) is transferred with a retroviral vector, into an aliquot of cells from TIL early in their culture, the marked cells are grown in parallel with unmarked cells, and both populations are reintroduced into the patient [see Armenato *et al.*, J. Virol. 61, 1647 (1987); Bender *et al.*, ibid, p. 1639; Miller *et al.*, Mol. Cell. Biol. 6, 2895 (1986)]. The anti-TNF-R2 antibodies of the present invention can be used in this protocol to promote TIL proliferation and/or to treat the patients following administration of the marked and unmarked TIL. Similarly, the antibodies herein may be useful in other gene marker protocols known in the art or under development, including a protocol using semiquantitative polymer chain reaction (PCR) to compare the amount of marked TIL in tumor tissue with the amount measured in neighboring normal skin and muscle.

A direct outgrowth from the NeoR/TIL gene marker protocol involves the transfer of the gene for TNF in order to make TIL more effective against advanced malignant melanoma. It is known that although TNF is a powerful anticancer agent, in humans its toxic effects become profound at relatively low doses (about 8 μg/kg/body weight). By transfecting a TNF gene into tumor infiltrating lymphocytes, and letting the TIL home to tumor deposits, it is believed possible to develop effectively high doses of TNF at tumor sites and avoid systemic side effects. [See Hum. Gene Ther. 1, 441 (1990)]. As the anti-TNF-R2 antibodies of the present invention only elicit a small spectrum of TNF activities, they are particularly advantageous for use in this protocol as a substitute for, or in addition to TNF.

The anti-TNF-R2 antibodies of the present invention may further find utility in the ADA gene transfer protocol. ADA is an enzyme that plays an important role in catalyzing purine metabolism. ADA deficiency has been traditionally cured by matched bone marrow transplantation. According to the ADA gene protocol [Hum. Gene Ther. 1, 327 (1990)], autologous lymphocytes transduced with a normal human ADA gene are administered to patients diagnosed with ADA-deficient severe combined immunodeficiency (SCID), in order to reconstitute the function of their cellular and humoral immune system. Patients are subjected to leukophoresis once a month to isolate the mononuclear cells by Ficoll gradient, and to grow these cells in culture under conditions that stimulate T lymphocyte activation and growth. At present, T-cell growth is simulated with OKT3 anti-CD3 antibody, and the cells are grown in IL-2. The anti-TNF-R2 antibodies of the present invention can be used for T lymphocyte growth stimulation alone, or in combination with OKT3, or any other stimulator

of T-cell proliferation. Once the T-cells are divided, they are incubated with a retroviral vector carrying a normal ADA gene, and preferably a marker (e.g. a NeoR gene), grown, and administered to the patient by infusion.

For review of the currently known human gene marking and gene therapy protocols see Anderson, W.F., Science 256, 808-813 (1992).

E. Analytical and Diagnostic Uses

The monoclonal antibodies of the present invention are suitable for detecting a human type 2 TNF receptor by contacting a source suspected to contain such a receptor with a detectably labeled monoclonal antibody herein, and determining whether the antibody binds to the source.

There are many different labels and methods of labeling known in the art. Suitable labels include, for example, enzymes, radioisotopes, fluorescent compounds, chemi- and bioluminescent compounds, paramagnetic isotopes.

TNF-R2 may be present in biological samples, such as biological fluids or tissues. For analytical or diagnostic purposes, the antibodies of the present invention are administered in an amount sufficient to enable the detection of a site on a TNF-R2 for which the monoclonal antibody is specific. The concentration of the detectably labeled monoclonal antibody should be sufficient to give a detectable signal above background, when bound to a TNF-R2 epitope.

The following example is offered by way of illustration only and is not intended to limit the invention in any manner.

EXAMPLE

Materials and Methods

Reagents. Recombinant human TNF and recombinant human LT, were cloned and expressed in *Escherichia coli* (specific activity 6.0×10^7 U/mg and 1.0×10^8 U/mg of protein respectively, Genentech, Inc.). The rabbit antibodies to human TNF-R1 (hTNF-R1) [Tartaglia, L.A. and Goeddel, D. V., *J. Biol. Chem.* 267, 4304-4307 (1992)] were generated against the soluble extracellular domain of hTNF-R1 and had a titer of 1:80,000 as quantitated by a direct antigen coated ELISA. The rabbit antibodies to human TNF-R2 (hTNF-R2) were generated against the soluble extracellular domain of hTNF-R2, and had a titer of 1:60,000 as quantitated by a direct antigen coated ELISA. The panel of 10 ascites purified monoclonal antibodies to human TNF-R2 (1035, 1036, 1037, 1038, 1040, 1044, 1045, 1046,1047, and 1048) were produced as described hereinbelow.

Production of anti-hTNF-R2 Monoclonal Antibodies - Twelve purified monoclonal Abs against human TNF-R2 (1035, 1036, 1037, 1038, 1039, 1040, 1043, 1044, 1045, 1046, 1047 and 1048) were produced by hyperimmunizing BALB/c mice in the hind footpads with

soluble TNF-R2 (sTNF-R2), the preparation of which is described hereinbelow, in RIBI adjuvant (RIBI ImmunoChem Research, Hamilton, MT) and fusing the draining inguinal and popliteal lymph node cells with the mouse myeloma cell line X63-Ag8.653 [Kearney, J.F. *et al.*, J. Immunol. 123, 1548-1550 (1979)]. The antibodies were purified from ascites fluid using protein A-Sepharose (Repligen Corp., Cambridge, MA) and established affinity chromatography methods [Goding, J.W., J. Immunol. Methods 20, 241-253 (1978)].

Characterization of Antibodies

A. **Epitope Mapping**

A competitive binding ELISA was used to determine whether the anti-TNF-R2 monoclonal antibodies bind to the same or different epitopes (sites) within hTNF-R2. [Kim, *et al.*, Infect. Immun. 57:944 (1989). Individual unlabeled anti-TNF-R2 monoclonal antibodies obtained as hereinabove described were added to the wells of microtiter plates that previously had been coated with hTNF-R2. Biotinylated anti-TNF-R2 monoclonal antibodies were then added. The ratio of biotinylated antibody to unlabeled antibody was 1:1000. Binding of the biotinylated antibodies was visualized by the addition of avidin-conjugated peroxidase, followed by o-phenylenediamine dihydrochloride and hydrogen peroxide. The color reaction, indicating the amount of biotinylated antibody bound, was determined by measuring the optical density (O.D) at 495 nm wavelength.

B. **Isotyping**

The isotypes of the anti-TNF-R2 monoclonal antibodies produced as hereinabove described were determined by ELISA. Samples of culture medium (supernatant) in which each of the hybridomas was growing were added to the wells of microtiter plates that had previously been coated with hTNF-R2. The captured anti-TNF-R2 monoclonal antibodies were incubated with different isotype-specific alkaline phosphatase-conjugated goat anti-mouse immunoglobulins, and the binding of the conjugated antibodies to the anti-TNF-R2 monoclonal antibodies was determined by the addition of p-nitrophenyl phosphate. The color reaction was measured at 405 nm with an ELISA plate reader.

C. **Binding Affinity**

The affinities of the foregoing anti-TNF-R2 monoclonal antibodies were determined by a competitive binding assay. A predetermined sub-optimal concentration of monoclonal antibody was added to samples containing 20,000 - 40,000 cpm ^{125}I-hTNF-R2 (1 - 2 ng) and various known amounts of unlabeled hTNF-R2 (1 - 1000 ng). After 1 hour at room temperature, 100 μl of goat anti-mouse Ig antisera (Pel-Freez, Rogers, AR USA) were added, and the mixtures were incubated another hour at room temperature. Complexes of antibody and bound protein (immune complexes) were precipitated by the addition of 500 μl of 6%

polyethylene glycol (PEG, mol. wt. 8000) at 4° C., followed by centrifugation at 2000 x G. for 20 min. at 4° C. The amount of ^{125}I-hTNF-R2 bound to the anti-hTNF-R2 monoclonal antibody in each sample was determined by counting the pelleted material in a gamma counter.

Affinity constants were calculated from the data by Scatchard analysis.

The properties of the anti-TNF-R2 antibodies used in the present assays are set forth in the attached Table.

Production of shTNF-R2 - The mammalian expression plasmid pRK5-sTNF-R2 consisted of an 862 bp fragment of TNF-R2 cDNA inserted between the cytomegalovirus immediate early promoter and the SV40 termination and polyadenylation signals of the vector pRK5 [Schall *et al.*, 1990, Cell 61, 361-370 (1990)]. The fragment contains 81 bp of 5' untranslated DNA and 771 bp encoding the 22 amino acid signal peptide and 235 amino acid extracellular domain of TNF-R2 followed by a stop codon that was inserted using synthetic DNA. The plasmid pRSV neo, containing a neomycin resistance gene, was cotransfected with pRK5-sTNF-R2 into the human embryonic kidney cell line 293 [Graham *et al.*, J. Gen. Virol. 36, 59-77 (1977)] by the calcium phosphate precipitation method [Gorman, C. in cDNA Cloning: A Practical Approach (Glover, D.M. ed.) 1985, Vol. II, pp. 143-190, IRL Press, Washington D.C.]. G418 resistant clones were isolated and tested for sTNF-R2 secretion by dot blot analysis [Heller *et al.*, J. Biol. Chem. 265, 5708-5717 (1990)] of the media with [^{125}I]TNF-α. A single clone, sTNF-R2-15, was found to secrete sTNF-R2 and was selected for further experiments.

A 0.5-1.0 liter sample of medium (F12 supplemented with insulin, transferrin, and trace elements) conditioned for 48 hr from confluent cultures of the sTNF-R2-15 cell line was concentrated approximately 75-fold by ultrafiltration through a 10,000 molecular weight cutoff YM-10 membrane. The concentrate was applied to a 1 x 5 mm TNF-α affinity column (human TNF-α coupled to CH-Sepharose 4B; Pharmacia). Bound protein was eluted from the affinity column with 0.2 M glycine (pH 3.0). Fractions were analyzed for sTNF-R2 by SDS-PAGE and then further purified by reverse-phase HPLC on a 4.6 x 100 mm Synchropak RP-4 column (Synchron, Inc.). The C_4 column was eluted at a flow rate of 0.5 ml/min with a gradient of mobile phase A [0.1% trifluoroacetic acid (TFA) in H_2O and mobile phase B (0.07% TFA in acetonitrile)]. Protein was monitored at 214 nm. The peak fractions (0.5 ml) were collected in tubes containing 0.3 ml of phosphate-buffered saline, pH 7.4 (PBS), lyophilized, and resuspended in water. Protein concentrations were determined by amino acid composition of aliquots.

Thymocyte Proliferation Assay. Human thymocyte sections were obtained from infants and very young children during cardiac surgery (Stanford University, Palo Alto, CA) and

cultured in 96-well flat-bottomed culture plates (6.0×10^5/ 0.1 ml or 0.05 ml) (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% gentamicin (Gibco Life Technologies, Grand Island, NY) in the presence of 2 μg/ml of Concavilin-A (Con A; Calbiochem, La Jolla, CA). Con-A, huTNF, and antibodies were added to a final volume of 0.2 ml. After 60 h at 37°C, cultures were pulsed with 1 μCi of [^{3}H]thymidine (5 Ci/mmol; 1 Ci = 37 BGq; New England Nuclear) for 12-18 h and harvested onto glass fiber filters (PhD; Cambridge Technology, Watertown, MA). Mean [^{3}H]thymidine incorporation (cpm) of triplicate cultures was determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA).

PBMC Proliferation Assay. Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood of healthy donors by Ficoll-Hypaque density sedimentation. The purified PBMC were washed three times in PBS and cultured in 96-well flat-bottomed plates (1×10^5 per 0.05 ml) (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% gentamicin (Gibco Life Technologies, Grand Island, NY) in the presence of a 1:1000 dilution of Phytohemagglutinin-P (PHA-P; Difco, Detroit, MI). PHA-P and antibodies were added to a final volume of 0.2 ml per well. Plates were incubated at 37° C for 5 days. Cultures were pulsed with 1μCi of [^{3}H]thymidine (6.7 Ci/mmol; ICN Biochemicals, Costa Mesa, CA) for the last 12-18 h and harvested onto glass fiber filters (PhD, Cambridge Technology, Watertown, MA). Mean [^{3}H]thymidine incorporation (cpm) of triplicate cultures was determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA).

Results and Discussion:

In support of previous reports [Reem, G. H., *et al.*, Cancer Research 49, 3568-3573 (1989)], TNF can significantly stimulate the proliferation of human thymocytes in the presence of the comitogen Con A (Fig. 1A). To determine which of the two TNF receptors mediates human thymocyte proliferation, we examined the ability of receptor specific polyclonal antibodies to signal this response. Polyclonal antibodies directed against human TNF-R1 had no effect on human thymocyte proliferation (Fig. 1B) despite the demonstrated ability of these antibody preparations to act as agonists of TNF-R1 and mimic a number of TNF effects (Tartaglia, L.A. and Goeddel, D. V., supra; Wong, G.H.W., *et al.*, Molecular Biology of Free Radical Scavenging Systems, Cold Spring Harbor Laboratory Press. 69-96 (1992); Wong, G.H.W. *et al.*, J. Immunol.

In press.]. However, polyclonal antibodies against TNF-R2 strongly stimulated thymocyte proliferation while preimmune sera had no effect (Fig. 1B). Similar results were obtained with thymocytes from at least three other individuals (see below; data not shown).

These results indicate that TNF induces human thymocyte proliferation via TNF-R2 and that antibodies against human TNF-R2 can act as receptor agonists. In addition, these data indicate that the two human TNF receptors, like the two murine TNF receptors, are both active in signal transduction yet mediate distinct cellular responses. Anti-human TNF-R1 antibodies have been shown to initiate signals for a large and diverse group of TNF activities including cytotoxicity, fibroblast proliferation, resistance to chlamidiae, synthesis of prostaglandin E2, NF-kB induction, MnSOD mRNA induction, and antiviral activity. In those cases examined, antibodies to TNF-R2 were not able to induce these responses. To our knowledge, thymocyte proliferation is the first example of a TNF response that can be initiated by an agonist to human TNF-R2 and not human TNF-R1.

Like TNF, lymphotoxin (LT) has also been shown to bind both TNF receptors with high affinity [Loetscher, H. *et al.*, supra; Schall, T.J. *et al.*, supra; and Smith, C A. *et al.*, supra] and to generate biological signals through TNF-R1. With the identification of human thymocyte proliferation as a TNF effect signaled by TNF-R2, we tested whether LT acts as an agonist or antagonist of this response. This question is particularly important in light of the many activity differences between TNF and LT that are still unexplained [Fiers, W. FEBS Lett. 285 (2), 199-212 (1991) and references therein]. As shown in Fig. 2, LT can potently stimulate the proliferation of human thymocytes. At low concentrations LT mediated proliferation was comparable to that of TNF and at high concentrations was somewhat better. The reduced proliferation seen at high concentrations of TNF (Fig. 2) has also been observed with the murine TNF stimulation of murine thymocyte proliferation (33). As predicted by the ability of LT to stimulate human thymocyte proliferation, LT did not antagonize the effects of TNF in this assay (data not shown). These results indicate that both TNF and LT can initiate a biological signal that is mediated by TNF-R2. Therefore, the explanation for the reported activity differences between LT and TNF is still not clear as both ligands appear capable of activating both receptors. It is possible that the functional differences between these two cytokines are a result of distinct orientations of receptor crosslinking resulting in qualitatively or quantitatively distinct signals.

As a first step toward identifying a therapeutically useful agonist of TNF-R2, we assayed a series of 10 monoclonal antibodies generated against TNF-R2 for potential agonist activity. A number of the anti-TNF-R2 mAbs exhibited a dose dependent stimulation of human thymocyte proliferation, while a control mouse mAb had no effect (Fig. 3). However, several mAbs showed either no or small effects even at high concentrations. To better distinguish those mAbs that do not possess agonist activity from those that possess low activity, thymocytes from several individuals were screened with high concentrations of each mAb.

These assays showed that although all thymuses tested were responsive to both TNF and anti-TNF-R2 mAbs, the degree of responsiveness varied from individual to individual. Fig. 4 shows data from the most responsive thymocytes tested. In this assay, 9 of the 10 anti-TNF-R2 mAbs resulted in a statistically significant stimulation of proliferation. Responses obtained with the 9 effective agonist antibodies varied from approximately 2-fold to greater than 6-fold. The maximal response seen with TNF in this assay (taken from a dose response run in parallel) was approximately 3.5 fold.

The ability of the large majority of mAbs against TNF-R2 to act directly as agonists is surprising. This has not been the case with TNF-R1 where agonist activity by the majority of mAbs requires either cross-linking with a second antibody or a combination of at least two mAbs [Engelman, *et al.*, J. Biol. Chem. 265, 14497-14504 (1990); Wong, *et al.*, Molecular Biology of Free Radical Scavenging Systems, Cold Spring Harbor Laboratory Press, 69-96 (1992); and our own unpublished observations]. It remains to be established whether this difference in mAb induced signaling is due to TNF-R2 being more readily activated by receptor dimerization (as opposed to trimerization) or, alternatively, TNF-R2 accepting greater flexibility in the orientation of receptor cross-linking. This flexibility of TNF-R2 may be provided by the 55 amino acid spacer between the transmembrane domain and the presumed cysteine rich ligand binding motifs in the extracellular domain, a region which is considerably shorter in TNF-R1.

To determine whether TNF-R2 mediated T-cell proliferation is restricted to the immature T-cells within the thymus, we also examined the effects of TNF receptor agonist antibodies on the proliferation of peripheral T cells. Here again polyclonal antibodies to TNF-R2 were found to significantly enhance PHA-P stimulated proliferation. Therefore, even circulating T-cells remain responsive to TNF-R2 signals. In the assay shown, no effects were seen with either anti-TNF-R1 antibodies or pre-immune sera. Interestingly, in identical PBMC assays with cells from several other donors, small effects were sometimes observed with anti-TNF-R1 polyclonal antibodies in addition to the much more consistent effects of anti-TNF-R2 antibodies. Small effects of anti-TNF-R1 antibodies in PBMC proliferation assays has also been described elsewhere [Gehr, G., *et al.*, J. Immunol. 149, 911-917 (1992)]. However, we cannot yet be certain whether this data is an indication that some cells proliferate in response to signals from both TNF receptors, or whether the small amounts of TNF-R1 induced proliferation stem from other cell populations, possibly even contaminating non-lymphoid cells.

The non-redundant signaling of the two TNF receptors offers hope that greater clinical specificity of TNF actions can be realized at the level of individual receptor activation. However, clinically useful TNF activities should be separable from TNF's toxic activities. Specific activation of TNF-R1 appears to activate the large majority of TNF activities and therefore it is questionable whether specific agonists of TNF-R1 will have greater clinical utility than TNF itself. In contrast, it is likely that specific TNF-R2 agonists will only elicit a small

spectrum of TNF activities, in particular immune regulation, and therefore may not show the toxicity that is characteristic of TNF.

Deposit of Materials

The following culture has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Hybridoma	ATCC No.	Date
9D4	HB 11148	7 October 1992
6F2	HB 11149	7 October 1992
1H9	HB 11150	7 October 1992.

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organism will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of one aspect of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of

the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustration that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

Table

MAB	ELISA CAPTURE	BLOCKS COMPLEX	BINDS COMPLEX	ISOTYPE	EPITOPE x-block	ASC. #
1H9	++	-	.++	IgG2A	4	1035
2B1	++	_	+	IgG2a	4	
2D6	+	.+	-	IgG1	4?	
2E8.	++	.+	.+++	IgG2b	1	1036
3D12	++	.+	.+/-	IgG1	4	1043
4B4	++	.++	-	IgG1	2	1037
4D8	++	.++	-	IgG2b	4	1044
5G7	++	.+	+	IgG2b	4?	
6D10	++	.++	-	IgG2b	4?	1045
6E1.	+	-	.+/-	IgG1	4?	
6F2	++	-	+	IgG2a	4	1046
8C1	++	.++	-	IgG1	4	1047
9B7	++	-	.++	IgG2b	3	1038
9D4	++	-	+	IgG2a	4	1048
9F12	++	.++	-	IgG2a	4	1039
9H9	++	.++	-	IgG2a	2	1040
	Where: OD>1= ++ 0.5-1 = +	Where: >50% = ++ 25-50 = + <25% = 1	Where: OD>2 = + ++ 1-2 = ++ .5-1 = + .25-.5 = +/-			

Claims:

1. A monoclonal antibody which specifically binds a human TNF-R2 receptor and exhibits T-cell proliferation stimulating activity.

2. The monoclonal antibody of claim 1 which is capable of stimulating the proliferation of human thymocytes.

3. The monoclonal antibody of claim 1 wich is capable of stimulating the proliferation of human peripheral T cells.

4. A monoclonal antibody which specifically binds to substantially the same human TNF-R2 epitope as that recognized by an antibody selected from the group consisting of monoclonal antibodies 1035, 1046 an 1048, produced by hybridoma cell lines ATCC Accession Nos. HB 11150, HB 11149, and HB 11148, respectively.

5. The monoclonal antibody of claim 1 which is capable of stimulating the proliferation of human thymocytes.

6. The monoclonal antibody of claim 5 having human thymocyte proliferation stimulating activity equal to or greater than native human tumor necrosis factor (hTNF).

7. An isolated nucleic acid encoding a monoclonal antibody according to any of claims 1-6.

8. A hybridoma cell line which produces a monoclonal antibody according to any of claims 1-6.

9. The hybridoma cell line designated 1H9, deposited under ATCC Accession No. HB 11150, producing monoclonal antibody 1035.

10. The hybridoma cell line designated 6F2, deposited under ATCC Accession No. HB 11149, producing monoclonal antibody 1046.

11. The ybridoma cell line designated 9D4, deposited under ATCC Accession No. HB 11148, producing monoclonal antibody 1048.

12. A bispecific antibody specific for a human TNF-R2 and for a T-cell surface protein.

13. The bispecific antibody of claim 12 in a hetero-multimer form.

14. The bispecific antibody of claim 13 wherein the T-cell surface protein is a CD antigen.

15. The bispecific antibody of claim 14 wherein the CD antigen is selected from the group consisting of CD1, CD2, CD3. CD4, CD5, CD6, CD8, CD11a, CD11b, CD11c, and CD18.

16. The bispecific antibody of claim 12 wherein the T-cell surface protein is an interleukin-2 receptor (IL-2R).

17. The bispecific antibody of claim 16 wherein the specificity for IL-2R is provided by an IL-2 amino acid sequence.

18. The bispecific antibody of claim 12 wherein the T-cell surface protein is a ganglioside.

19. The bispecific antibody of claim 18 wherein the ganglioside-specificity is from an antibody to the G_{D3} ganglioside.

20. The bispecific antibody of claim 13 which comprises an Fc domain having an immunoglobulin effector function.

21. The bispecific antibody of claim 20 which comprises an Fc domain of IgA, IgD, IgE, IgG or IgM.

22. A method for stimulating human T-cell proliferation comprising administering to a human patient in need of such treatment a physiologically effective amount of a monoclonal antibody which specifically binds a human TNF-R2.

23. The method of claim 22 wherein said monoclonal antibody is bispecific.

24. The method of claim 22 wherein said monoclonal antibody is coadministered with a further T-cell mitogen.

25. The method of claim 24 wherein said further T-cell mitogen is IL-2.

26. The method of claim 22 wherein the patient has been diagnosed with a physiological condition selected from the group consisting of malignant tumors, T-cell mediated autoimmune disorders, immunodeficiencies, HIV, graft-versus-host disease, and potential of allograft rejection.

27. The method of claim 26 wherein the administration is parenteral.

28. The method of claim 27 wherein the administration is at a dosage of about 0.0001 mg/kg to about 1000 mg/kg.

29. The method of claim 27 werein the monoclonal antibody is labeled.

30. The method of claim 29 wherein the administration is by gene transfer.

31. A method for stimulating T-cell proliferation comprising growing a T-cell culture in the presence of an effective amount of a monoclonal antibody which specifically binds a human TNF-R2.

32. The method of claim 31 wherein the T-cells have been isolated from a malignant tumor of a human patient.

33. The method of claim 32 wherein the T-cells are tumor-infiltrating lymphocytes (TIL).

34. The method of claim 33 wherein the T-cell culture is grown until expansion of at least thousand times.

35. The method of claim 34 wherein the expanded T-cells are reintroduced into the patient from which they have been taken.

36. The method of claim 33 wherein a gene encoding said monoclonal antibody is introduced into said T-cells.

37. The method of claim 36 wherein said introduction is by a retroviral vector.

38. The method of claim 37 wherein the T-cells have been isolated from the blood of a human patient diagnosed with ADA deficiency, purine nuclease phosphorylase (PNP) deficiency, or hypoxanthine-guanine phosphoribisyltranserase (HGPRT) deficiency.

39. The method of claim 38 wherein the T-cells are transfected with a etroviral vector carrying a normal ADA, PNP or HGPRT gene.

40. A method for detecting a human TNF-R2 which comrpises contacting a source suspected of containing said receptor with a detectably labelled monoclonal antibody which specifically binds a native human TNF-R2, an determining whether the antibody bnds to the source.

FIG. 1A

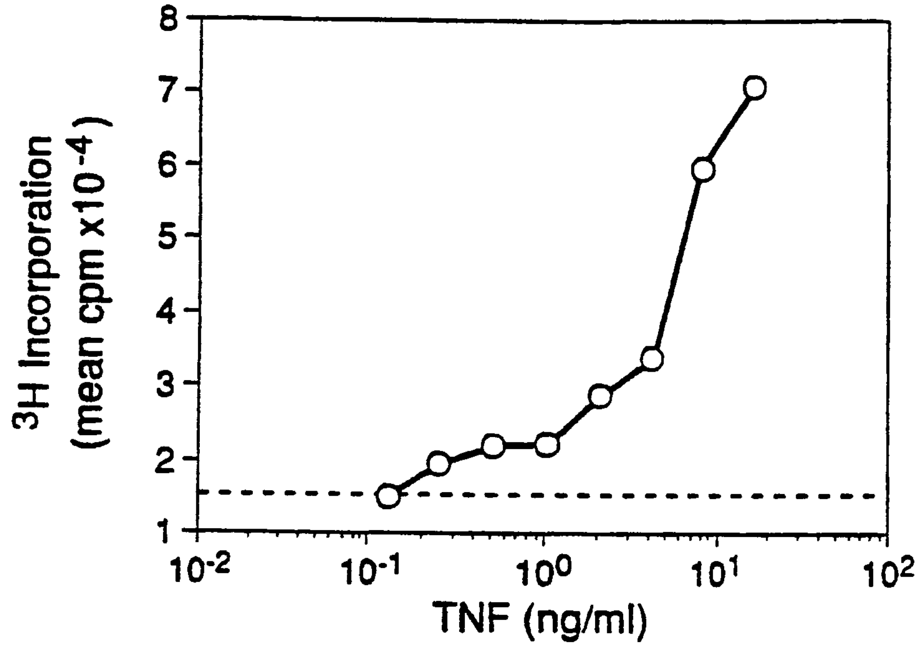

8
7
6
5
4
3
2
1
3H Incorporation
(mean cpm x10-4)
10-2
10-1
100
101
102
TNF (ng/ml)

FIG. 1B

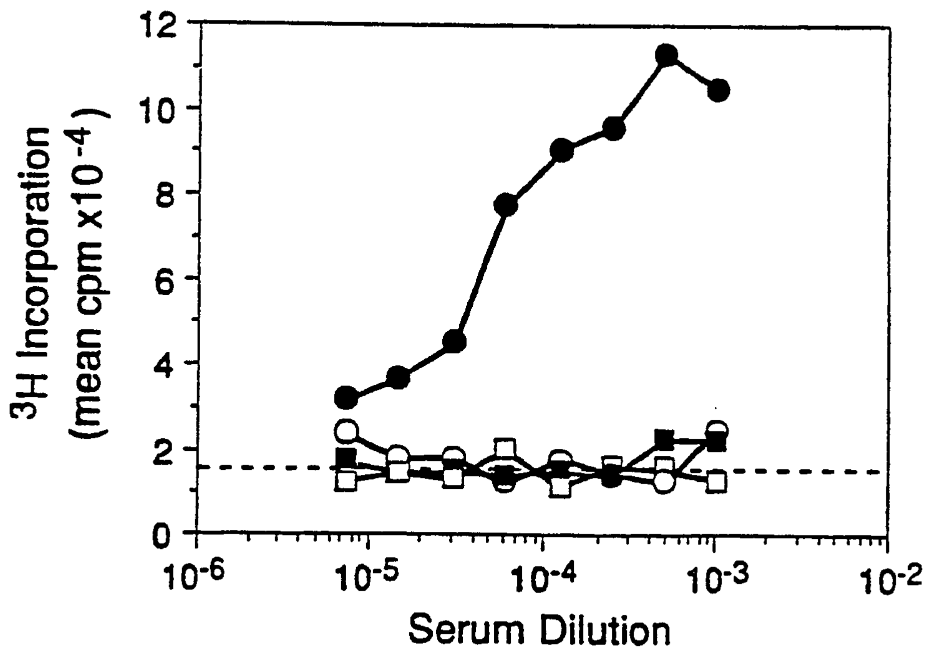
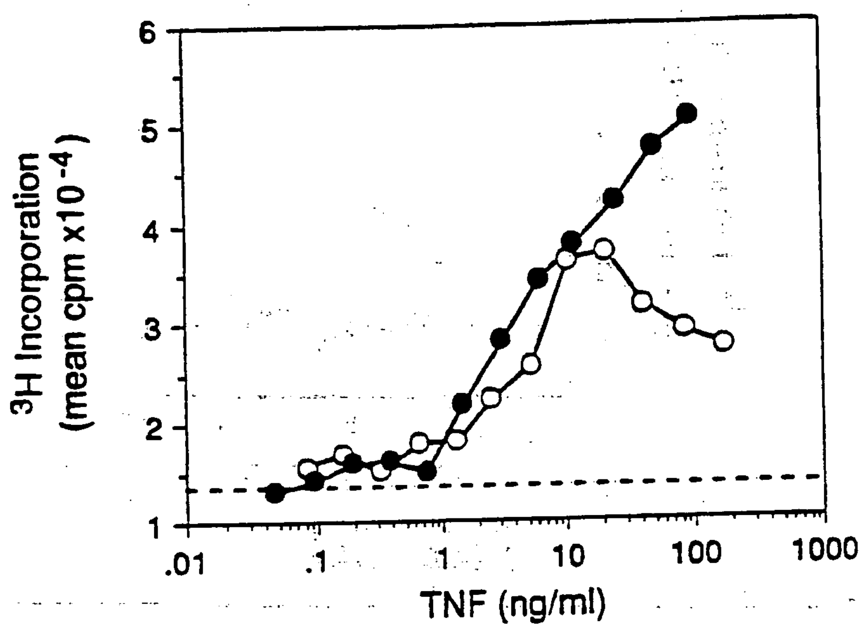
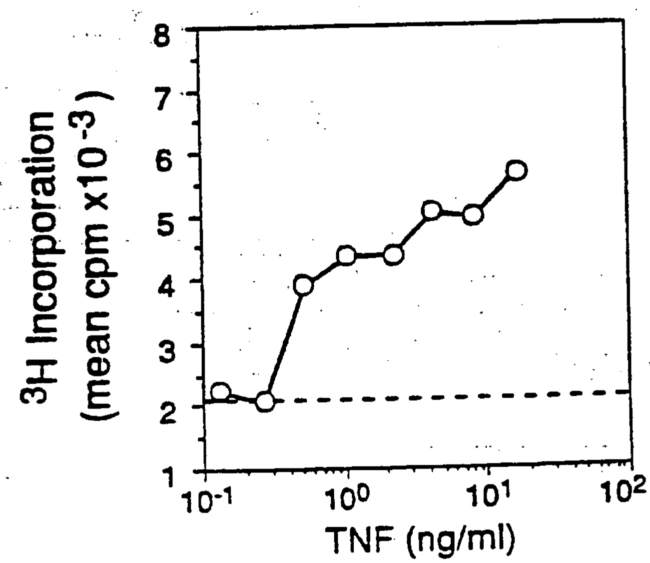

12
10
8
6
4
2
0
3H Incorporation
(mean cpm x10-4)
10-6
10-5
10-4
10-3
10-2
Serum Dilution

FIG. 2



3H Incorporation (mean cpm x10-4)
1
2
3
4
5
6
.01
.1
1
10
100
1000
TNF (ng/ml)

FIG. 3A



3H Incorporation (mean cpm x10-3)
1
2
3
4
5
6
7
8
10-1
100
101
102
TNF (ng/ml)

FIG. 3B

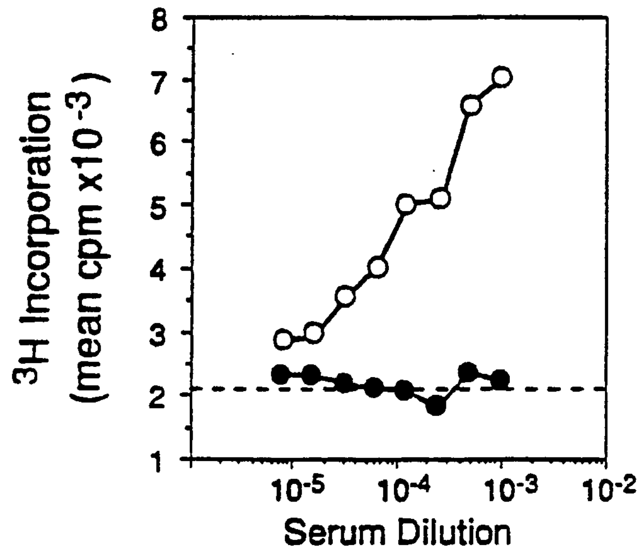

3H Incorporation
(mean cpm x10-3)
8
7
6
5
4
3
2
1
10-5
10-4
10-3
10-2
Serum Dilution

FIG. 3C

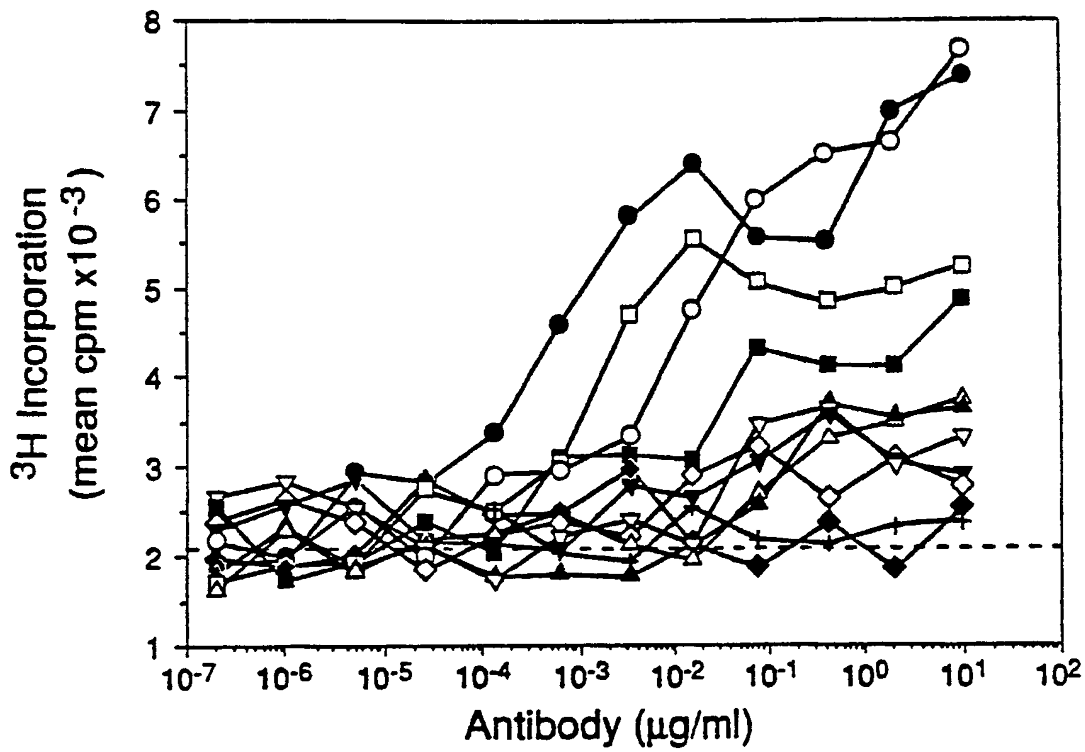

3H Incorporation
(mean cpm x10-3)
8
7
6
5
4
3
2
1
10-7
10-6
10-5
10-4
10-3
10-2
10-1
100
101
102
Antibody (µg/ml)

FIG. 4

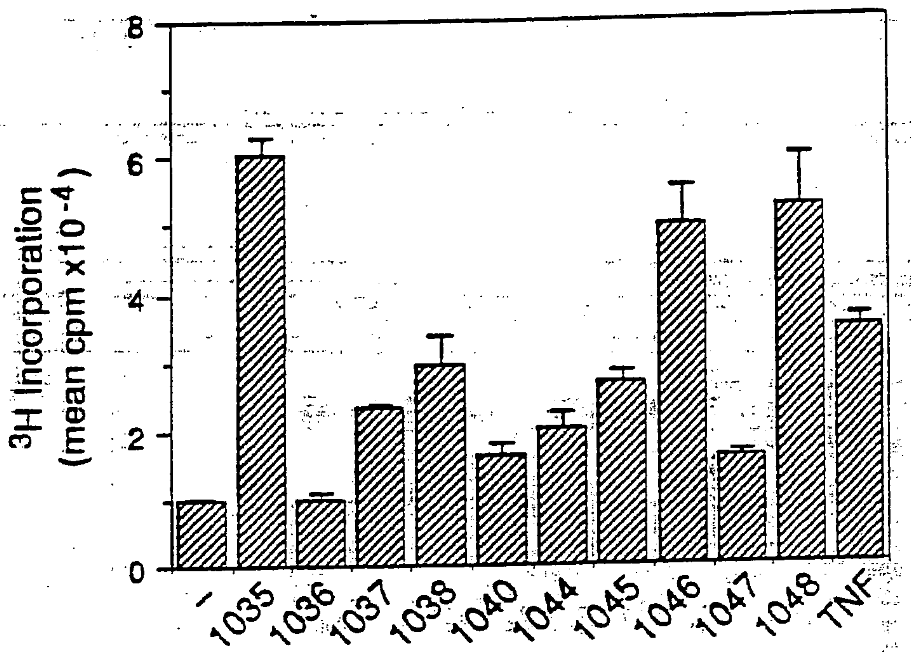
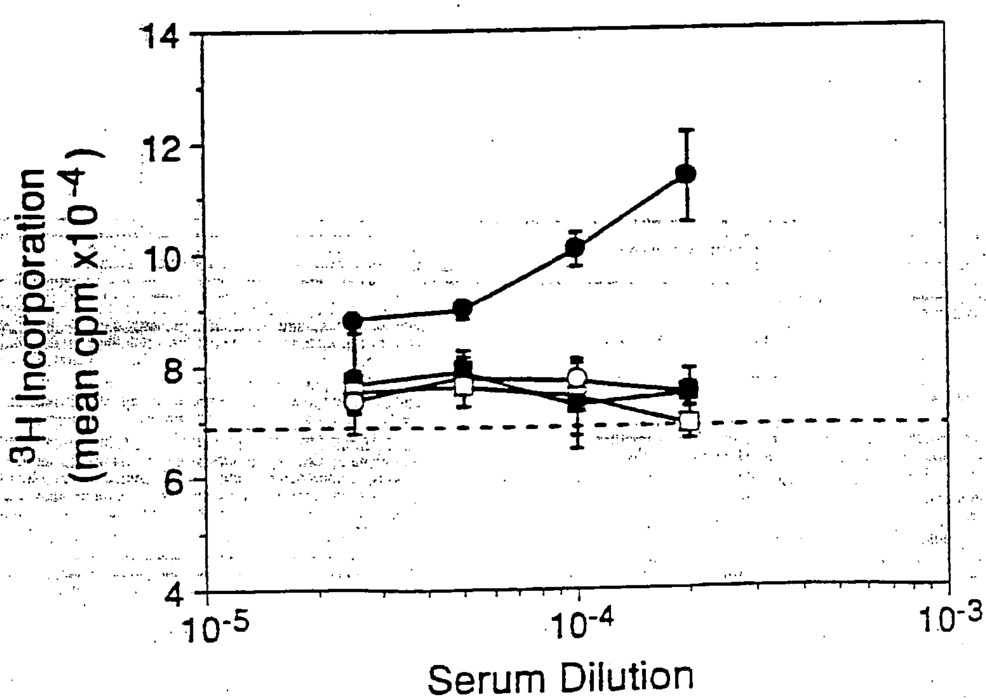

3H Incorporation (mean cpm x10-4)
0
2
4
6
8
-
1035
1036
1037
1038
1040
1044
1045
1046
1047
1048
TNF

FIG. 5


3H Incorporation (mean cpm x10-4)
4
6
8
10
12
14
10-5
10-4
10-3
Serum Dilution

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